Tissue Factor Pathway Inhibitor Attenuates the Progression of Malignant Pleural Mesothelioma in Nude Mice

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Malignant pleural mesothelioma (MPM) is a rare cancer that is refractory to current treatments. It is characterized by a robust deposition of transitional fibrin that is in part promoted by tumor cells. MPM cells express tissue factor (TF) and the tissue factor pathway inhibitor (TFPI), but their contribution to the pathogenesis of MPM has been unclear. We found that REN MPM cells fail to express TFPI. Based on the tumor growth–promoting properties of TF, we hypothesized that the stable transfection of TFPI into REN MPM cells would decrease their aggressiveness. We tested our hypothesis using *in vitro*, *in vivo*, and *ex vivo* analyses. TFPI knock-in decreased the proliferation, invasion, and TF activity of REN cells *in vitro*. REN TFPI knock-in cells, empty vector, and naive control cells were next injected intrapleurally into nude mice. The expression of TFPI significantly decreased tissue invasion, inflammation, and the deposition of fibrin and collagen associated with tumor tissue, pleural effusions, and tumor burden. In *ex vivo* analyses, REN cells were cultured from harvested tumors. The overexpression of TFPI was maintained in cells propagated from TFPI knock-in tumors, and attenuated the activation of Factor X and the invasiveness of tumor cells. These analyses demonstrate that TFPI reduces the aggressiveness of MPM *in vitro* and *in vivo*, and that its effect involves the inhibition of TF procoagulant activity. These observations suggest that the interactions of TF and TFPI represent a novel therapeutic target in the treatment of MPM.

Keywords: malignant pleural mesothelioma; tissue factor pathway inhibitor; proliferation; invasion; tumorigenesis

CLINICAL RELEVANCE

Our observations suggest that the fibrin neomatrix associated with malignant pleural mesothelioma may be important for tumor progression, and that the expression of tissue factor pathway inhibitor (TFPI) can attenuate tumor aggressiveness *in vitro* and *in vivo*. This work may also be clinically relevant because TFPI has the potential to be therapeutic against this neoplasm.

Malignant pleural mesothelioma (MPM) is a relatively rare cancer in which tumors originate from the pleural mesothelium. This cancer is associated with exposure to asbestos, and asbestos-challenged mesothelial cells can undergo malignant transformation over a period of several decades (1). Current treatments for this relatively rare form of cancer are unsatisfactory, and the survival rate for MPM is approximately 16 months from time of diagnosis (2). Consequently, new, more effective treatment strategies are mandatory and are being sought.

MPM is characterized by disordered fibrin turnover, and is associated with aberrant depositions of fibrin (3, 4). These depositions occur at least in part because of decreased local fibrinolysis by the urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) and the coagulation initiated by tissue factor (TF). As fibrinogen is released from the vasculature, it is rapidly clotted because of the TF-mediated amplification of the coagulation cascade. Thus, TF favors the formation of a transitional fibrinous neomatrix, which characterizes a number of solid malignancies and may contribute to their growth and aggressiveness (5). We recently showed that the urokinase receptor (uPAR) promotes the aggressiveness of MPM, and that invasive tumors are associated with florid extracellular fibrin, suggesting that factors promoting transitional fibrin deposition could influence tumor aggressiveness. We previously showed that human MPM cells express two such factors (i.e., TF and TFPI, both *in vitro* and *in situ* in resected and autopsy specimens) (3).

The role of TF, TFPI, and the extravascular deposition of fibrin in the pathogenesis of MPM remains unclear, and represents a potentially important gap in current knowledge. In many cancers, the dysregulation of TF expression occurs during tumorigenesis (6, 7). The overexpression of TF in cancer cells was found to be closely correlated with the deposition of fibrin (8). The increased expression of TF is associated with higher tumor grades (greater aggressiveness) and angiogenesis (9, 10), which promotes their growth and invasiveness (11). TF is a 47-kD transmembrane glycoprotein that initiates the extrinsic coagulation cascade during inflammation or neoplasia (5, 6). Direct signaling from TF could be responsible for the increased angiogenesis of cells that overexpress TF (10, 12). In melanoma cells, the overexpression of TF was also reported to contribute to increased tumor growth and metastasis (10, 13–15). TF signaling also plays an important role in tumor progression (16, 17). On the other hand, tissue factor pathway inhibitor (TFPI) is the key inhibitor of TF activity. TFPI is a 42-kD tridomain protein that binds to the TF, Factor VIIa, and Factor X complex, suppresses the generation of Factor Xa by TF, and impedes ongoing coagulation. TFPI blocks angiogenesis and metastasis *in vitro* and *in vivo* (11). We hypothesized that TFPI is a particularly critical determinant of the growth and invasiveness of MPM and of the extravascular fibrin we previously

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found to be associated with the tumor (4). In this study, we discovered that MPM cells that lack TFPI (REN cells) were more aggressive. Based on these considerations, we sought to elucidate the role of TFPI in the growth of MPM *in vivo*. We tested our hypothesis using *in vitro*, *in vivo*, and *ex vivo* approaches. We found that TFPI decreased the proliferation, invasion, and TF-dependent activation of Factor X in TFPI knock-in REN cells. Using an *in vivo* orthotopic model of MPM in nude, athymic mice, we similarly found that tumor burden was significantly decreased by the overexpression of TFPI in injected REN cells, and that tumor cells propagated from the harvested masses retained their expression of TFPI and the same *in vitro* indices of attenuated aggressiveness.

MATERIALS AND METHODS

Creation of Stable TFPI-Expressing MPM Cells

REN MPM cells were engineered to stably express increased amounts of TFPI. REN cells were stably transfected with the pcDNA 3.1 empty vector (EV; Invitrogen, Carlsbad, CA) or TFPI-1 (TFPI) cDNA. Two days after transfection, cells were selected in RPMI complete media containing G418 (400 μg/ml; Invitrogen). Individual clones were then selected and expanded. Clones were assayed for their increased expression of TFPI. Please see the online supplement for additional details.

Interventions in the Orthotopic MPM Murine Model

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler. REN MPM cells were prepared for intrapleural injection into nude athymic mice (BALB/c athymic NCr-*nu/nu*; National Cancer Institute, Frederick, MD) as previously described (4). Naive, EV, and TFPI-expressing REN MPM cells were grown to confluence. Cells were then lifted using trypsin, washed with PBS, and counted. We resuspended 1.5×10^6 cells in 150 μl of a PBS/Matrigel mixture. Cells were then loaded into a chilled 25-gauge syringe and kept on ice. Ten mice per group were allocated for the intrapleural administration of naive, EV, and TFPI-expressing REN cells. Injections were scheduled over a period of 3 days. Ten animals, involving three to four mice from each group, were injected each day. The mice were maintained for 25 days and weighed weekly and on the day during which they were killed. Of 10 mice per group, three were dedicated to histologic and immunohistochemical analyses of the thoracic cavities. Tumors were isolated from the remaining seven cavities and dedicated to Western blotting and the propagation of tumor cells.

Measurement of Lung Mechanics

Pulmonary function tests were used to determine the effects of MPM tumor burden on lung function. Mice were anesthetized with a ketamine/xylazine mixture. After anesthesia, intubation was performed by inserting a sterile 18-gauge intravenous canula through the vocal cords into the trachea. A computer-controlled piston ventilator (flexiVent system; Scireq, Tempe, AZ) was used to ventilate each mouse mechanically through the canula, using a tidal volume of 30 ml/kg at a frequency of 150 breaths/minute and 2–3-cm H_2O positive end-expiratory pressure. These data were then applied to calculate elastance, compliance, and total lung resistance, using a single-compartment model.

Computed Tomography Scans and Measurements of Lung Volume

While ventilated, isoflurane gas was use to ensure that mice remained deeply anesthetized to minimize spontaneous breaths. The Explore Locus Micro-CT Scanner (General Electric) was used for computed tomography (CT) imaging. Microview software was used to analyze lung volumes and render three-dimensional images. Images were assessed at a constant window level. The rendered images were used to calculate lung volumes at total lung capacity.

RESULTS

TF and TFPI Antigen Analyses in MPM Cells

The expression of TF and TFPI was initially assessed in REN, MS-1, and M9K MPM cells by Western blotting (Figure 1). The expression of TF was markedly increased in REN versus MS-1 and M9K MPM cells. TFPI was detected in the lysates and conditioned media (CM) of MS-1 and M9K cells. Moreover, no TFPI antigen was detectable in the lysates or conditioned media of REN cells (data not shown).

TFPI Reduces *In Vitro* Markers of Tumor Aggressiveness

Because REN cells expressed increased TF and no detectable TFPI, we tested the ability of TFPI to alter the aggressiveness of MPM. REN cells were engineered to overexpress TFPI. The expression of TF was relatively unaffected by the overexpression of TFPI in REN cells (Figure 2A). We next tested the functionality of stably expressed TFPI in REN cells, using FXa conversion assays. Naive and EV-expressing REN cells demonstrated a comparable ability to activate FXa in these analyses (Figure 2B). TFPI-expressing REN cells demonstrated significantly reduced conversion of FXa, indicating that the TFPI was functional and blocked the cell-surface activity of TF. The addition of an antibody to human TFPI to the samples reversed the inhibition of Factor X–activating activity in these samples (data not shown).

[^{3}H]thymidine incorporation assays were next performed to determine the effects of TFPI expression on the proliferation of REN cells. TFPI–REN cells proliferated to a significantly reduced extent, compared with naive and EV-expressing REN cells (Figure 2C). Parallel cell-count assays demonstrated the same reduced proliferation of TFPI REN cells versus control cells (not shown). In Matrigel invasion assays, naive, EV, and TFPI-expressing REN cells were seeded on Matrigel invasion chambers and allowed to migrate toward an FBS gradient for 12–15 hours. TFPI-expressing REN cells exhibited decreased invasiveness compared with naive and EV-expressing REN cells (Figure 2D). In the aggregate, these studies indicate that the expression of TFPI reduces the aggressiveness of REN cells *in vitro*.

Expression of TFPI Decreases MPM Tumor Burden in Nude Mice

The ability of TFPI to reduce the proliferation and invasion of REN cells *in vitro* provided a strong rationale to test its effects

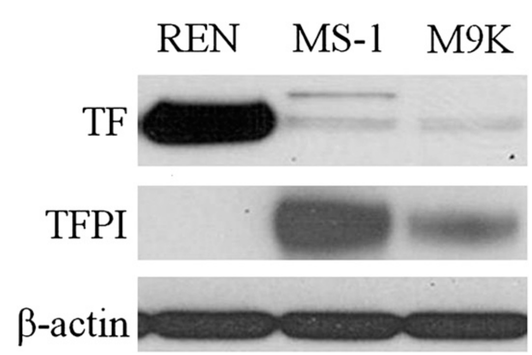

Figure 1. Expression of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in REN, MS-1, and M9K malignant pleural mesothelioma (MPM) cells. (*A*) Serum-starved cells were lysed, and 50 μg of cleared cellular lysate were resolved via SDS-PAGE and analyzed by Western blot analysis. The expression of β-actin was assessed as the loading control.

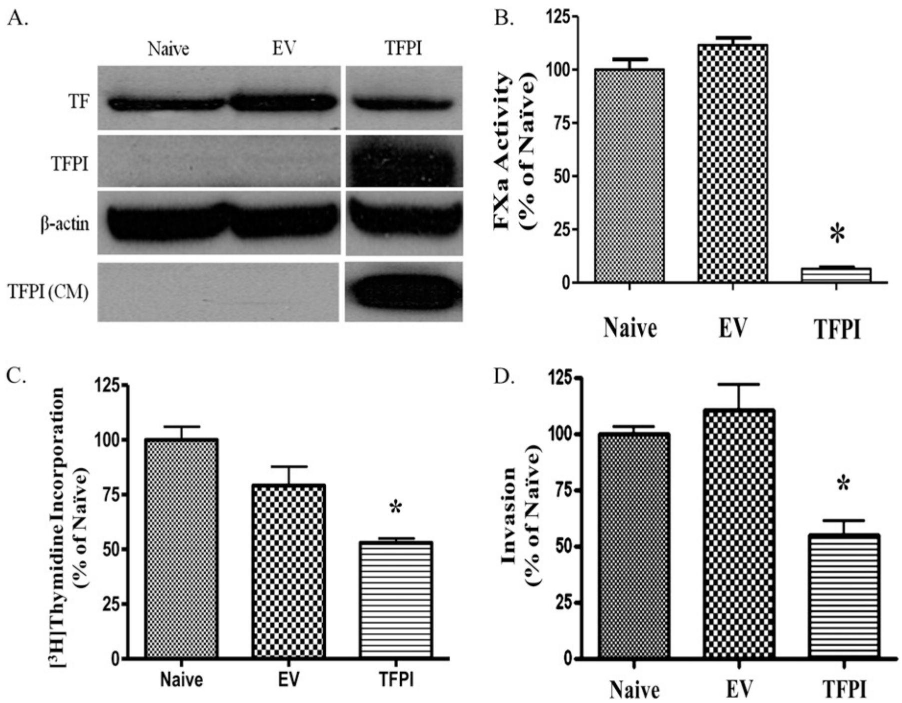

Figure 2. TFPI-expressing REN cells exhibit reduced conversion, proliferation, and invasion of Factor Xa (FXa). (*A*) Western blot analysis shows that the TFPI transfectants express more TFPI than do naive and EV cells in conditioned media (CM) and cell lysates. (*B*) The cell-surface TF activity of naive, EV, and TFPI-expressing REN cells was assayed with an FXa conversion assay. TFPI-expressing REN cells generated less Factor Xa than did naive and EV-expressing REN cells. *$P < 0.05$, compared with naive or EV REN cells. (*C*) Serum-starved naive, EV, and TFPI-expressing REN cells were treated with 1 μCi/ml of [^{3}H]thymidine for 12 hours in serum-free RPMI media. TFPI-expressing REN cells proliferated to a lesser extent than did naive and EV-expressing REN cells. *$P < 0.05$, compared with naive or EV REN cells. (*D*) TFPI-expressing REN cells displayed decreased invasion compared with naive and EV-expressing REN cells. *$P < 0.05$, compared with naive and EV REN cells.

in vivo, so we injected naive, EV, and TFPI-expressing REN cells intrapleurally into nude mice. Because one naive REN-injected and one EV tumor–bearing mouse died on Day 24, the animals were killed at 25 days after intrapleural injection. Naive, EV, and TFPI-tumor–bearing mice were subjected to CT scanning to visualize their tumor masses *in vivo*. TFPI-expressing REN cells produced few or no detectable tumor masses and no pleural densities, compatible with effusions by CT scan (Figure 3A). Conversely, naive and EV–REN tumor–bearing mice typically demonstrated large tumor masses and pleural densities compatible with effusions or overlying tumor masses (nine evaluable mice in the naive and EV groups; Figures 3B and 3C). From three-dimensional renderings of CT images, lung volumes were next calculated. The naive and EV REN cells produced large tumor masses that caused significant reductions in lung volume ($P < 0.05$) compared with TFPI-tumor–bearing mice (Figure 3D).

We also performed pulmonary function tests to determine the effects of injecting TFPI-expressing REN cells on lung elastance, compliance, and resistance in comparison to naive and EV cells. No significant differences were evident between the three experimental groups (data not shown). These studies indicate that although intrapleural injections of REN cells produced large tumor masses, they did not affect the compliance, elasticity, and resistance of the underlying lung, and these indices of lung parenchymal physiology were spared from the adverse effects of tumor burden that were largely extrinsic to the lung.

We next killed the naive, EV, and TFPI-tumor–bearing mice, and directly assessed their tumor burden. At the time of killing, pleural effusions were collected and quantified. TFPI tumor–bearing mice did not produce detectable pleural effusions, whereas the naive and EV-tumor–bearing mice all produced 300–500 μl of bloody, pleural effusion ($n = 9$ in both the naive and EV groups). Tumors were then counted, measured, and weighed. TFPI-expressing REN cells produced significantly fewer tumors that the naive and EV-expressing REN cells (Figure 4A). Furthermore, only four out of 10 TFPI-expressing REN cell–inoculated animals demonstrated detectable tumors. The TFPI-expressing tumors were also significantly smaller in volume and weight (Figures 4B and 4C). These data confirm that the expression of TFPI reduces the emergence of REN tumors *in vivo*.

Assessment of MPM Tumor Aggressiveness

Because we previously reported that REN MPM cells produced highly invasive tumors with a florid deposition of fibrin (4), thoracic cavities ($n = 3$ per group) were collected from the naive, EV, and TFPI-tumor–bearing mice groups and assessed for invasiveness, inflammation, and fibrin or collagen deposition. On gross inspection, tumors of TFPI-tumor–bearing mice ($n = 4$) (Figure 5C) were generally smaller than in the naive (Figure 5A) and EV (Figure 5B) tumor-bearing mice. Tissue sections were prepared from each group and assessed for tumor invasion. Naive (Figure 5D) and EV-expressing (Figure 5E) REN cells formed tumors that invaded the skeletal muscle of the parietal pleura, and were characterized by an increased deposition of collagen within the tumors versus those generated by TFPI-expressing REN cells (Figure 5F). Moreover, inflammatory infiltrates were typically prominent within the tumor masses in the naive and EV-expressing tumors, but were not evident in the TFPI–REN–generated tumors (Figure 5I). Immunohistochemical analysis showed that the deposition of fibrin was less prominent within TFPI-expressing tumors (Figure 5I) than in either naive (Figure 5G) or EV-expressing (Figure 5H) tumors. In all cases, the deposition of fibrin was relatively more pronounced at the tumor surface, possibly reflecting that the tumors were bathed in bloody-appearing pleural fluid. Tumor sections were also immunostained for the expression of TFPI (Figure 5M). Prominent expression of TFPI was only evident in TFPI-expressing REN tumors. Cytospin analyses of naive and EV-tumor– bearing murine pleural lavages were characterized by the presence of fibrinous material and lymphocytes and small, blue-staining cells (Figure 5N). Pleural lavage cytospins of TFPI-tumor–bearing animals lacked fibrinous material but contained lymphocytes and MPM cells, some of which were in clusters. This finding suggests that TFPI-bearing REN cells remained viable and may have dehisced from the pleural

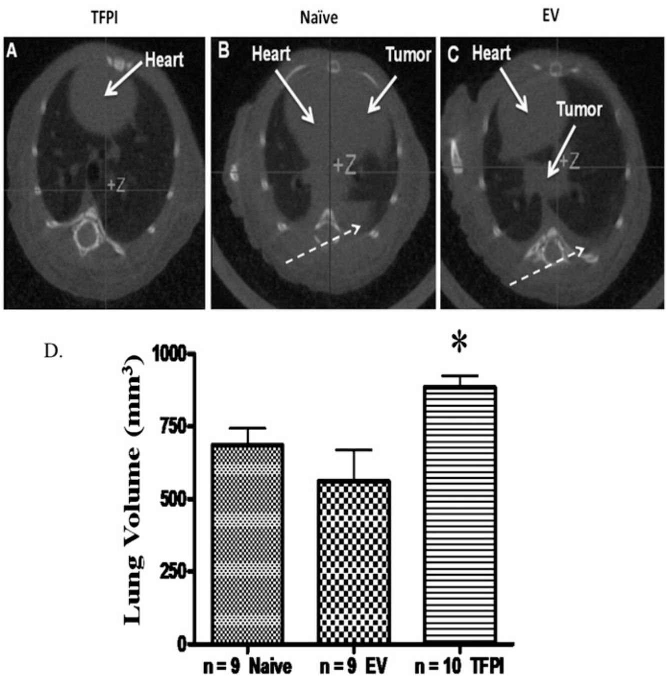

Figure 3. TFPI-expressing REN cells demonstrate reduced tumor burden *in vivo*. Computed tomography (CT) scans were performed on live mice that had been injected with naive, EV, and TFPI-expressing REN cells 25 days earlier. Representative CT scans show that TFPI-expressing REN cells (*A*) produced no detectable tumors in this example, whereas naive (*B*) and EV-expressing (*C*) REN-injected mice developed detectable exophytic tumors. *Broken arrows* indicate a pleural-based mass $\pm$ effusion (naive example, *B*) and a pleural effusion (EV in *C*). CT scans were used to render three-dimensional reconstructions of tumor-bearing murine lungs, using Microview software. (*D*) TFPI-tumor bearing mice exhibited significantly larger lung volumes than did naive and EV tumor–bearing mice. Z = sagittal plane marker. *$P < 0.0.5$, compared with naive and EV tumors.

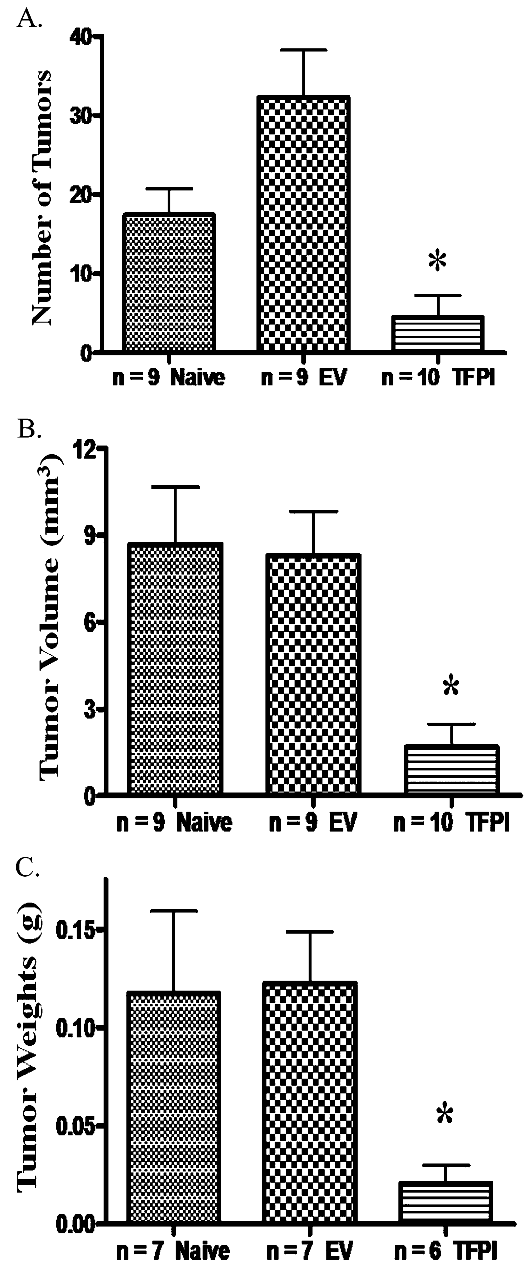

Figure 4. The expression of TFPI reduced the size and number of REN tumors. (*A*) Naive, EV, and TFPI- expressing REN tumor–bearing mice were killed, and their tumors were counted. TFPI-expressing, tumor-bearing animals had significantly fewer tumors than did naive and EV tumor–bearing mice. EV tumor–bearing mice produced significantly more tumors than did naive tumor-bearing mice. Naive, EV, and TFPI-expressing tumors were also measured using a digital vernier caliper. Volumes were calculated and are presented as average total volume per animal. (*B*) TFPI-expressing tumors are significantly smaller than naive or EV-expressing REN tumors *$P < 0.05$, compared with naive and EV tumors. Naive, EV, and TFPI-expressing tumor cells were collected, weighed, and averaged per animal. (*C*) TFPI-expressing tumors weighed significantly less than naive and EV-expressing tumors. *$P < 0.05$, compared with naive and EV tumors.

surface, where they generally failed (in 6/10 mice) to form discrete tumor masses. These data collectively show that the intrapleural injection of TFPI-expressing REN cells produced less invasive tumors that contained less collagen and fibrin than tumors formed by naive and EV-expressing REN cells.

Pleural Lavage Findings and *Ex Vivo* Analysis of Propagated Tumors

Because the TFPI-expressing REN cells produced fewer, smaller exophytic tumors, we sought independent confirmation that the expression of TFPI was increased *in vivo*. The pleural cavities were therefore lavaged with 1 ml of saline, collected, cleared via centrifugation, and assayed by Western blot analysis for TFPI antigen. TFPI antigen was consistently detected (9/10 mice) only in pleural lavages from TFPI-expressing cell–injected mice (n = 9 for naive and EV mice, and n = 10 for TFPI REN–challenged mice). TFPI antigen was detectable in the lavage of one EV and one naive mouse, possibly reflecting the contribution of nonmalignant mesothelial cells or fibroblasts in this animal. A representative Western blot analysis of the pleural-lavage TFPI findings is depicted in Figure 6A. These findings indicate that TFPI-expressing cells continued to produce TFPI throughout the 25-day inoculation period.

Tumors were also isolated and prepared for *ex vivo* analysis. Tumors from naive, EV, and TFPI-expressing REN tumors were diced and cultured on 100-mm tissue culture dishes. We were able to culture MPM cells successfully from five naive, four EV, and three TFPI–REN–generated tumors. Cultured tumor cells were also assessed for the mesothelial markers calretinin and cytokeratin 7. The propagated tumors consisted of

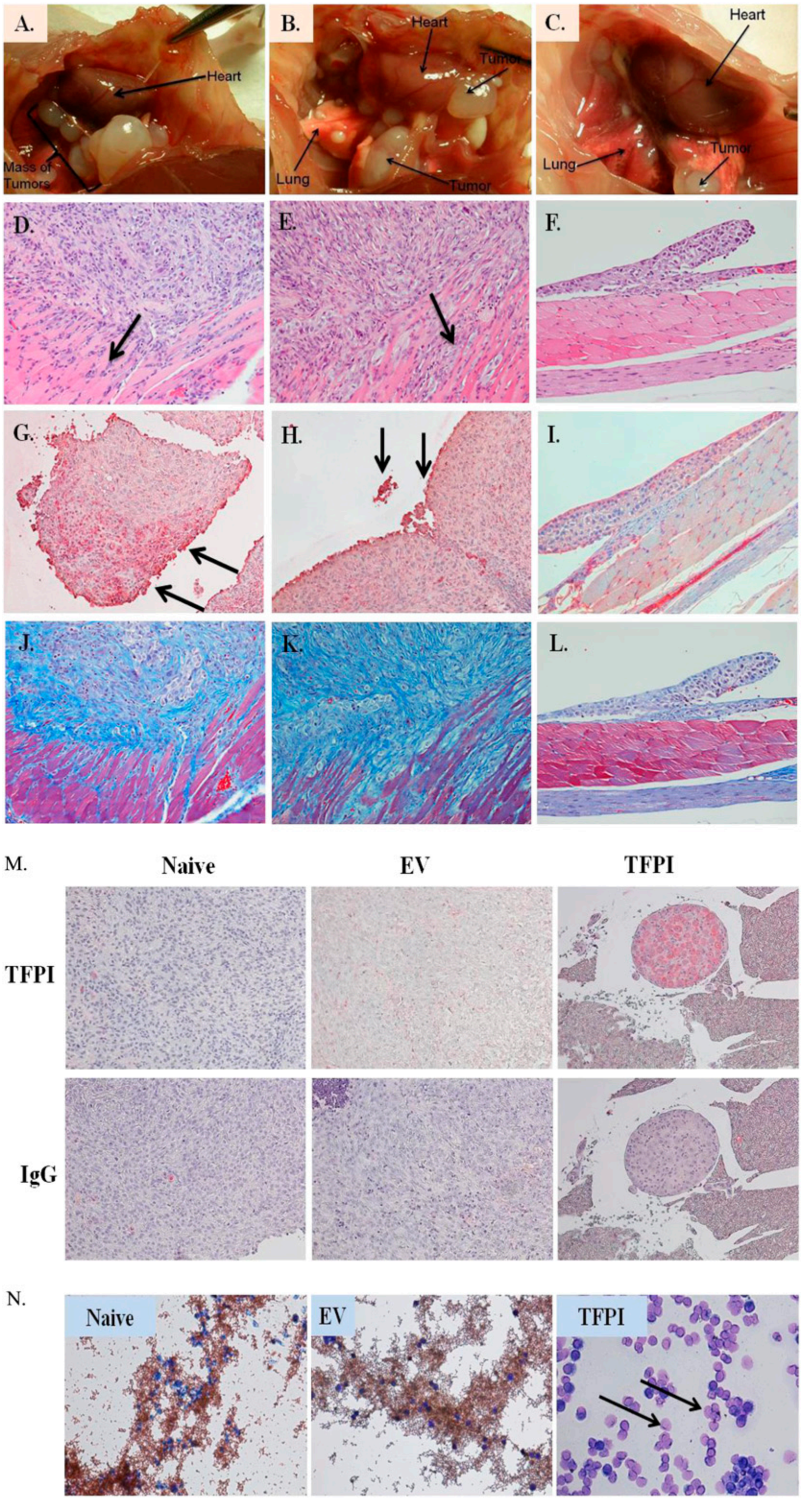

Figure 5. TFPI-expressing REN tumors are less invasive and are characterized by less fibrin and collagen deposition. On gross inspection, naive (*A*) and EV (*B*) tumors produced larger exophytic tumors than TFPI-expressing REN cells, when tumors were detected in this group (4/10 mice, *C*). Representative tumor sections were prepared from naive (*D*), EV (*E*) and TFPI-expressing (*F*) MPM tumor-bearing mice, and were trichrome stained. Naive and EV-expressing tumors were invasive as shown by invasion of the skeletal muscle of the parietal pleura in representative sections. Conversely, the TFPI-REN tumors were not invasive and relatively little collagen was detectable within the tumors (*F*). Tumor sections from the pleural cavities of naive (*G*), EV (*H*) and TFPI-expressing (*I*) tumor-bearing mice were next immunostained for mouse fibrin. TFPI-expressing REN tumors were associated with less apparent fibrin deposition than naive and EV-expressing REN tumors. *Solid arrows* indicate fibrin deposition at the periphery of the tumors, but staining was typically more diffuse in the naive and EV groups and increased compared to the TFPI-REN–generated tumors. MPM tumor sections from naive (*J*) and EV-expressing (*K*) tumor-bearing mice also stained positively for collagen expression at the sight of tumor invasion. (*I*) TFPI-expressing REN tumor-bearing mice expressed little collagen at the tumor site. Lastly, tumors sections from the naive, EV and TFPI mice were immunostained for TFPI (*M*). The naive and EV tumors expressed relatively little or no TFPI in the tumors compared to the TFPI-REN–generated tumors, in which TFPI staining in the tumor was prominent. (*N*) Pleural lavages from naive, EV and TFPI-expressing tumor-bearing mice were cytocentrifuged and analyzed. Lavages from naive and EV tumor-bearing mice displayed fibrinous material which contained lymphocytes (stained *blue*). Lavages from TFPI–expressing tumor-bearing mice contained no fibrin and MPM cells that clustered and recapitulated the appearance of the malignant cells within the tumors. *Solid arrows* indicate MPM cells. All images are representative of the findings that were consistently observed in the thoracic tissues from three mice from each group.

virtually all (>95%) MPM tumor cells according to our analysis of cytopreparations of the cells. These analyses confirmed that the harvested naive, EV, and TFPI-expressing tumor cells retained mesothelioma cell markers (data not shown). Sufficient numbers of cells were propagated during five passages to test confluent cells for the expression of TFPI and to measure their activity. Serum-starved cells were then tested for the expression of TFPI protein in tumor cell lysates and of TFPI in the conditioned media. TFPI was only detected in the TFPI-expressing tumor cell lysates and conditioned media (data not shown). Because TFPI antigen was detected in samples from TFPI-expressing tumor cells, we assessed the activity of TFPI in naive, EV, and TFPI-expressing REN tumor cells via FXa conversion analysis. TFPI-expressing cells demonstrated a significantly reduced ability to convert Factor X to FXa, compared with naive and EV-expressing tumor cells (Figure 6B). These

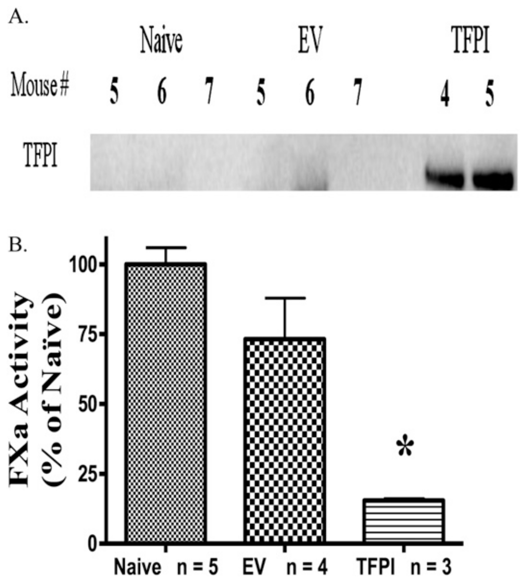

Figure 6. The expression of TFPI is increased in pleural lavages of mice injected with TFPI–REN cells, and its function is retained in cells harvested from these animals. Pleural lavages were performed on the pleural cavities of naive, EV, and TFPI-expressing REN cell–inoculated mice. The lavages were then collected, cleared, and subjected to Western blotting for the expression of TFPI. (*A*) TFPI was only detected in pleural lavages of mice inoculated with TFPI-expressing REN cells. Tumor cells were isolated, diced, and cultured on tissue culture dishes. Cell lysates were then prepared, and the conditioned media were collected from the cultured naive, EV, and TFPI-expressing REN tumor cells. Conditioned media and tumor-cell lysates were then subjected to Western blotting for TF and TFPI. β-actin was used as a loading control. (*B*) Harvested and propagated TFPI-expressing tumor cells demonstrated significantly less FXa conversion than did naive and EV-expressing tumor cells. $^*P < 0.05$, compared with naive and EV cells. Cells from two TFPI mice were compared with three mice per group in the EV and naive groups. All analyses were performed in duplicate.

analyses indicate that the harvested TFPI-expressing tumor cells retained the ability to express TFPI, and that the TFPI expressed was sufficient to reduce surface TF activity.

DISCUSSION

Our previous findings that the deposition of fibrin is florid in the MPM of mice and in patients with MPM (3, 4), and that both TF and TFPI are expressed in human MPM tissue (3), justify our focus on the role of TFPI in the pathogenesis of this disease. The increased expression of TF in tumor cells was associated with aspects of tumor progression, including angiogenesis, invasion, and metastasis (9, 10, 12). Both TF and TFPI are readily detectable in virtually all forms of MPM, including the epithelioid and sarcomatous (spindle-shaped) variants that occur in human disease (3). TFPI was reported to inhibit the growth of melanoma tumors in mice by more than 80% (13). It also significantly reduced the formation of lung metastases (13). Therefore, TFPI can influence the growth and propagation of some forms of malignancy. Although the role of TFPI in the pathogenesis of MPM was not, to our knowledge, previously studied, we hypothesized that TFPI could influence the progression of this tumor, and tested that possibility here.

TFPI occurs in two major forms. TFPI-1 is a TF inhibitor that is mainly responsible for inhibiting coagulation. Peritumoral injections of TFPI-1 in B16 melanoma tumors inhibited tumor growth (11). Because of the prominent deposition of fibrin we previously observed in association with the development of REN tumors in mice and in patients with MPM (3, 4), we studied the effects of TFPI-1 on the progression of MPM. REN cells are well suited for these analyses, because they form aggressive tumors and they do not express TFPI. On the other hand, TFPI-2 was discovered and named after TFPI-1 because of the similarities in their structures. TFPI-2, however, is a serine protease inhibitor that mainly functions as a plasmin inhibitor, and it weakly inhibits the coagulation cascade (11). Many studies were performed on TFPI-2 as an antiangiogenic and antimetastatic agent (11). Our results show that TFPI-1 can regulate the aggressiveness of MPM *in vitro* and *in vivo*. In future studies, we will study the role of TFPI-2 in the growth and invasiveness of MPM, using the same approach deployed in this project.

In humans, MPM is strongly associated with aggressive local growth, late metastasis, pleural effusions, focal inflammation, and the deposition of fibrin (1). These attributes were likewise found in the nude, athymic murine model we used. The deposition of fibrin was florid in tumors from the naive and EV groups, indicating that the model recapitulates this aspect of clinical MPM (3). This finding is especially impressive, because the pleural cavities were washed with saline, which might have disrupted at least some of the fibrinous material that occurs *in vivo*. Pleural effusions were also prevalent in the naive and EV mice. TFPI–MPM–bearing mice lacked pleural effusions, which paralleled the paucity of exophytic tumors and likely reflects the absence of pleural invasion. Interestingly, the pleural washes from mice challenged with TFPI-overexpressing REN cells demonstrated increased amounts of MPM cells that retained the characteristics of those seen within the tumors. In the majority of TFPI–REN mice, overt tumors did not form, but cells likely to be REN cells rather than reactive mesothelial cells were found in relative abundance within pleural washes. This result suggests that TFPI–REN cells did not readily form tumors *in vivo* and that they persisted and may be prone either to associate with loosely, or dehisce from, the pleural surface.

The REN cell line was shown to be more aggressive than the MS-1 and M9K MPM cell lines that express TFPI (4). Our *in vitro* experiments indicate that the invasion and surface activity of TF in live cells was decreased when TFPI was transfected in the REN cell line. Our results further show that the transfection of TFPI into REN cells decreases tumor growth and invasion after injection *in vivo*. Over the 25-day period allocated for tumor growth *in vivo*, the naive and EV-generated tumors proved more invasive and aggressive than those of the TFPI cells. In lung CT images, lung volumes were significantly reduced by the formation of exophytic tumors. Pulmonary function testing revealed that the formation of exophytic tumors did not affect the compliance or elastance of the lungs, and did not detectably affect airway resistance. Elastance and compliance are physiologic indices of the expansive capacity and stiffness of the lung parenchyma, which were unchanged by the growth of exophytic tumors. These results suggest that lung volume, as measured with Microview software to create a three-dimensional image of the lungs, is a more sensitive index of tumor growth external to the murine lung. Our data suggest that the TFPI knock-in mice manifested relatively larger lung volume because their pleural tumor load was relatively reduced versus the EV or naive mice.

The results further show that the administration of TFPI–REN cells resulted in decreased tumor counts, volumes, and weights versus the control EV or naive REN tumor groups. Although the EV tumor counts were significantly different from those in naive-injected mice, tumor volumes and weights were comparable in the naive and EV groups. These findings suggest that the transfection of REN cells affects their ability to coalesce as exophytic tumors, but that overall tumor burden was not changed in the EV group. The data also show, however, that tumor burden was significantly increased in the EV and naive groups versus the TFPI knock-in REN group.

An increase was readily apparent in the invasiveness of naive and EV-expressing tumors versus the TFPI group. Further, naive and EV-expressing tumor invasion was characterized by focal chronic inflammation and an extensive deposition of collagen, mirroring the histologic findings that may occur during MPM in humans (1). TFPI-expressing tumors were also found to be smaller, fewer in number, lacking in inflammatory cell infiltration, and not invasive. Tumor invasiveness correlated with the formation of sanguineous or bloody effusions and the increased formation of fibrinous neomatrix in the EV and naive groups. When grossly detectable, TFPI tumors were characterized by the formation of discrete, smaller tumors (in 4/10 mice) that presented no evidence of invasion *in vivo*. Furthermore, increased TFPI was found in the pleural lavage of mice challenged with TFPI–REN cells, indicating that the injected cells continued to express TFPI throughout the 25-day course of the experiments. The trend toward tumor formation in these mice parallels the *in vitro* trends we observed before the tumors were administered. Interestingly, the same trends were evident in tumor cells harvested and propagated from harvested tumors and then studied *in vitro*. These pathophysiologic trends were consistent with the durable retention of the TFPI gene product in TFPI knock-in cells from tumor-bearing mice, and strongly suggest that the expression of TFPI was responsible for the effect.

MPM is refractory to conventional cancer treatments. Our data strongly implicate TFPI in controlling the growth of MPM, extending our previous finding that TFPI is expressed in clinical tumor samples from patients with MPM (18). Because TFPI has already been used in clinical trials of patients with sepsis and pneumonia (19–21), the findings assume potential translational applicability. Our observations suggest that the administration of exogenous TFPI could restrict the progression of MPM. Because TFPI is no longer available in the quantities needed for preclinical interventional administration, testing this postulate would entail the production and characterization of recombinant TFPI. In future preclinical analyses, we will test the ability of exogenous TFPI to slow or reverse the growth of established MPM.

Author disclosures are available with the text of this article at www.atsjournals.org.

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